Indian Journal of Geo-Marine Sciences
Vol. 41(1), February 2012, pp. 76-82

Effect of *Excoecaria agallocha* leaves against *Aeromonas hydrophila* in marine ornamental fish, *Amphiprion sebae*

Dhayanithi N B, T T Ajith Kumar* & T Balasubramanian
Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University
Parangipettai-608 502, Tamil Nadu, India
*[E.mail: tt_ajith@yahoo.co.in]

Received 28 December 2010; revised 05 February 2011

Present study was performed to validate the potential of mangrove, *Excoecaria agallocha* leaves against *Aeromonas hydrophila* isolated from infected marine ornamental fish, *Amphiprion sebae*. Infected ten fish samples were collected from the marine ornamental fish hatchery and the bacteria was isolated and identified as *Aeromonas hydrophila*. Antibiotic resistance characterization was performed with commercial antibiotics. *In vitro* antibacterial activity of methanol and acetone extract of *Excoecaria agallocha* and their minimum inhibitory concentration (MIC) were assessed. Healthy *Amphiprion sebae* when challenged with the *Aeromonas hydrophila*, resulted in the development of clinical signs, those observed in the hatchery. The methanol extract of *Excoecaria agallocha* leaf was administrated with live *A. hydrophila,* decreased the percentage of mortality in the experimental groups. The phytochemical screening of the extract showed the presence of alkaloids, phytosterols, fixed oils and fats, tannins, phenolic compounds, proteins, free amino acids, gums, mucilage, flavonoids and lignin. The results revealed that the *Excoecaria agallocha* leaves have the potential to control the *Aeromonas hydrophila* diseases in marine ornamental fish, *Amphiprion sebae*.

[**Keywords** : Mangrove, fish, hatchery, diseases, aquaculture, *Excoecaria agallocha*]

Introduction

Marine ornamental fishes are called as "living jewels" on account on their beautiful colors and playful behaviors[1]. The commercially important marine ornamental fishes are clowns, damsels, angels, wrasses, butterflies and sea horses. Clown fish (Anemone fish) belongs to the family Pomacentridae is one of the most popular marine fish highly suitable for home aquaria.

In Asian countries, the fish culture continues to be ravaged by bacterial diseases such as motile aeromonad septicaemia (MAS), furunculosis and edwardsiellosis. Among these, MAS caused by *Aeromonas hydrophila* is most widespread in fishes[2]. *A. hydrophila* is a ubiquitous, opportunistic free-living Gram-negative bacterium prevalent in crowded aquatic habitats[3]. MAS infects number of organisms producing stress related diseases with the common symptoms of ulcerations, exophthalmia and abdominal distension[4,5,6]. The use of antibiotics and chemotherapeutics for prophylaxis and treatment in intensive aquaculture has been widely criticized for their negative impacts like accumulation of drugs in tissues, development of drug resistance and immunosuppression[7]. Alternatively, for some diseases, vaccines against specific pathogens have been developed with varying degree of success. The wide range of pathogens in fish farming also limits the effectiveness of vaccine[8]. Hence, there is an urgent need to look for ecofriendly disease preventative measures to promote sustainable ornamental aquaculture.

Mangrove is a natural resource for tannin and the timbers produced are of great value. However, little is known on the antioxidant potentials[9]. Mangrove plants are used in folklore medicine to treat various diseases for centuries[10]. Some of the mangrove plants have been screened for their antiviral, antibacterial, antiulcer and anti-inflammatory activities[11]. The wood, latex and leaves contain alkaloids and terpenoids among which some posses anticarcinognic activity[12]. Roots of the plant served as a source of chlorine-containing diterpenes agallochines A-C, and of a secondary derivative of agallochine. However, little is known on the antibacterial and antifungal properties of *E. agallocha* against fish pathogens.

Materials and Methods

Isolation and identification of bacteria

The moribund fishes of *Amphiprion sebae* were collected from the marine ornamental fish hatchery, Annamalai University. Dead fishes were collected

from the tanks and morphologically examined for symptoms and microscopically examined for the presence of parasites and protozoan. The liver, kidney and spleen of the freshly dead fishes were aseptically removed, homogenized and serially diluted up to 10^{-5} to 10^{-8} and plated on Zobell marine agar 2216 (ZMA) and nutrient agar (NA) (Hi Media, Mumbai) with 50% seawater. After incubation at 28°C for 48 hours, predominant bacterial colonies were pure cultured and identified[13].

Antibiotic susceptibility assay

Antibiotic susceptibility tests were carried out against the isolated bacterial strains with 8 antibiotics by disc diffusion method[14]. Antibiotic discs of Ampicillin (30 µg), gentamicin (30 µg), penicillin G (30 µg), streptomycin (30 µg), erythromycin (30 µg), oxytetracycline (30 µg), tetracycline (30 µg) and chloramphenicol (30 µg) were used for the study. Twenty milliliter of Muller Hinton Agar (MHA) medium were poured into the plates to obtain uniform depth and allowed to solidify. The standard inoculum suspension (10^6 cfu/mL) was swabbed over the surface of the media using sterile cotton swab to ensure the confluent growth. The discs were placed on the surface of the plate with sterile forceps and pressed gently to ensure contact with the inoculated agar surface. Finally, the inoculated plates were incubated at 37°C for 24 h and the inhibition zones were observed including the diameter of the disc (5 mm). All the experiments were carried out in triplicate.

Collection of mangrove plant leaves

Fresh leaves of the mangrove plant, *Excoecaria agallocha* were collected from Vellar estuary, Cuddalore District, Tamil Nadu, India. After shade drying, the leaves were powdered by using mechanical grinder[15]. Five grams of the dry powder were extracted in 50 mL of methanol and acetone using a rotary shaker for 30 min at 100 rpm and the supernatant was separated by centrifugation at 10,000 rpm for 30 min. After centrifugation the supernatant portion was evaporated and crude extract has stored in refrigerator for further studies.

Antibacterial activity

Antibacterial activity of *E. agallocha* leaves was tested against the isolated bacteria by disc diffusion method[16]. The antibacterial susceptibility tests were performed on Muller–Hinton Agar medium. The standard inoculum suspension (10^6 cfu/ml) was streaked over the surface of the media using sterile cotton swab to ensure the confluent growth of the organism. The 5 mm diameter discs were prepared with Whatman No.1 paper. About 500 µg of crude extract was impregnated on the filter paper discs. The discs were placed on the surface of the plate with sterile forceps and pressed gently to ensure contact with the inoculated agar surface. Erythromycin (30 µg/disc) was used as positive reference standard to determine the sensitivity of the tested strains and PBS was used as negative control. The inoculated plates were incubated at 37°C for 24 hrs and the inhibition zones were observed including the diameter of the disc (5 mm). All the experiments were carried out in triplicate.

Assessment of minimum inhibitory concentration

The minimum inhibition concentration (MIC) of methanol and acetone extracts of *E. agallocha* leaves was determined by broth dilution assay method[17]. The inoculation of the bacterial strains were prepared from 12 hr old broth cultures and suspensions were adjusted to standard turbidity (10^6 c.f.u./ml). Crude extract of *Excoecaria agallocha* was dissolved in Phosphate buffer saline (PBS) to obtain 2,000 µg/ml stock solution. About 0.5 ml stock solution was incorporated into 0.5 mL of Muller–Hinton broth to make concentrations of 1000, 500, 250, 125, 62.5, and 31.25 µg/ml. The stock concentration was changed again and again for analysis of MIC value. Fifty microliter of standard suspension of the test organism was transferred to each test tube. The *Excoecaria agallocha* leaves extract and PBS was used as control. After 24 hr of incubation, the results were evaluated by reading at 620 nm in spectrophotometer.

Determination of bioactive compounds

Preliminary phytochemical compounds were analyzed by the chemical methods[18] to find out the presence of alkaloids, carbohydrates, glycosides, phytosterol, oils, fats, saponins, tannins, phenolic compounds, proteins, free amino acids, gums, mucilages, flavonoids, lignin and volatile oils.

Fish collection and acclimatization

Healthy sub-adults (100 Nos.) of common clownfish, *Amphiprion sebae* were obtained from (10-15 g in weight) the marine ornamental fish hatchery and transferred to 2500 l capacity water holding cement tank filled with UV treated seawater.

The fishes were maintained in the same tank for 4 days with continuous aeration and fed with boiled oyster meat twice a day. The water quality parameters during the experiment were, water temperature 28.2°C ± 1.4, *p*H 8.2 ± 0.3, salinity 28 ± 2.2 PSU, dissolved oxygen 7.8 ± 0.6. Ammonia and nitrite in the water were below detectable levels.

Experimental infection

After acclimatization, active fishes were shifted to experimental tank 200 l FRP tank with 150 l UV sterilized seawater under controlled conditions. The bacterial culture was centrifuged at 1000 g for 10 min at 4°C. The supernatant were discarded and the bacterial pellet was washed three times and resuspended in phosphate-buffered saline (PBS) at pH 7.4[6]. The OD of the solution was adjusted to 0.5 at 456 nm which corresponded to 1×10^6, 1×10^7 & 1×10^8 c.f.u ml^{-1} and the same concentrations of *A. hydrophila* pellet was dissolved in experimental groups of 10 fishes each in triplicates along with PBS. Since, the experimental fishes were small in size, intraperitoneally injection method was not adopted. Control group were exposed only PBS without *A. hydrophila*. Once the fish had been exposed with bacterial isolate, the fishes were observed in every day for mortality. The clinical symptoms were noted including hemorrhagic septicemia of the body surface.

Disease resistant test

Healthy Clownfish, *A. sebae* (n = 10) were fed along with 1, 10 and 50 mg of methanol extract powder in 2% body weight of the fish on day 1 and the control fish (n = 10) was fed without mangrove extract. In the experimental tank were exposed to a 24 h culture of *A. hydrophila* in different tanks as pellet form with final concentration of 1×10^7 colony forming units (cfu) under aseptic conditions on day 4. The challenge dose was standardized as 1×10^7 (c.f.u.) ml^{-1} by earlier conducted challenge study and the same concentration was exposed to the experimental groups the control group was exposed to the same amount of buffer with absence of bacteria[19].

Results

The infected common clownfish *A. sebae* expressed haemorrhagic septicemia in body surface (Fig. 1). Based on the microscopical examination of gill and body surfaces, no parasites and protozoan was observed. Different numbers of same bacterial colonies were predominately isolated from the immune organs of the diseased fishes. Results from the culture characteristics indicated that the bacterial isolate from the infected fishes are exhibiting the typical aeromonad morphological and physiological characteristics, such as mucoid yellow colored colonies on GSP plates, buffo colored on Tryptic soya agar, dark green color round colonies on *Aeromonas* isolation medium, gram negative, rod-shaped bacteria, cytochrome oxidase positive, D-glucose fermentation positive, arginine dihydrolase positive, ornithine decarboxylase negative, ONPG positive, H_2S from cystein, acetoin from glucose, gas from glucose, l-arabinose utilization and fermentation of salicin were confirmed that they belongs to *Aeromonas hydrophila*. This bacterium has previously isolated from other marine ornamental fishes in the same hatchery and identified[20]. In the drug susceptibility assays (Table 1), the isolated bacterial strain were resistant against Chloramphenicol (10 mm), oxytetracycline (9 mm), tetracycline (11 mm) and sensitive to erythromycin (20 mm), penicillin G (18 mm), streptomycin (18 mm) and trimethoprim (20 mm). Methanol and acetone extracts of *E. agallocha* leaves exhibited sensitive (20 mm) and intermediate (10 mm) against the isolated *A. hydrophila* respectively. In the antimicrobial activity, results indicated that methanol extract exhibited maximum activity (20 mm) than acetone extract (13 mm) (Fig. 2). Methanol extract at a concentration of 500 µg has recorded the minimum inhibitory concentration (MIC) against the *A. hydrophila* (Table 2). In the challenge experiment, challenge dose was standardized as 1×10^7 colony forming units (c.f.u.) ml^{-1} to give 100% mortality in

Fig. 1—Clinical symptoms of *Amphiprion sebae* infected by *Aeromonas hydrophila*

Table 1- Antibiotic susceptibility assay with commercial antibiotics

Commercial antibiotics (30μg)	Susceptibility results (mm)	Result
Chloramphenicol	10	R
Erythromycin	20	S
Oxytetracycline	09	R
Penicillin G	18	S
Streptomycin	18	S
Tetracycline	11	R
Trimethoprim	20	S

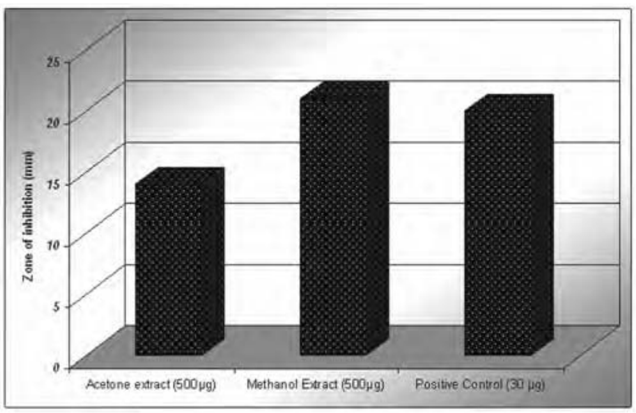

Fig. 2—Antibiotic activity of *Excoecaria agallocha* leaves against *A. hydrophila*

the untreated group. Mortality was recorded on day 2 to 4. The clinical symptoms were noted including hemorrhagic septicemia, distended abdomen, skin muscles with faded pigments and lesions on the ventral surface of the body. The cause of death was confirmed by re-isolating the organism from liver & kidney using *Aeromonas* isolation medium. On day 4, post methanol extract treated fishes were challenged with *A. hydrophila* and cumulative mortality was registered during 10 days in the diseases resistant experiment. The percent mortality of significantly reduced to 40% and 30% where, the fish fed with 1 and 10 mg kg^{-1} of methanol extract. The percent mortality was untreated control group (0 mg kg^{-1}) was 90%. The highest dose of 50 mg kg^{-1} methanol extract showed higher percent mortality at 50%. The percent mortality was significantly decreased in the methanol solvent extract treated fish, when challenged with live *A. hydrophila* (Fig. 3). Among the groups administered with single dose of methanol solvent extract, the doses of 1 and 10 mg kg $^{-1}$ gave the maximum protection with a percent mortality of 45% and 30%, followed by 50% mortality in the group treated with 50 mg kg^{-1} extract. The untreated group (0 mg kg $^{-1}$) showed high percent mortality of 90%. The enhancement of non-specific immune parameters by methanol solvent is possibly an important factor in reducing the percent mortality and thereby protecting the fish against live *A. hydrophila* challenge. Methanol extract of *E. agallocha* leaves showed the presence of alkaloids, phytosterols, fixed oils, fats, tannins, phenolic

Table 2 - Minimum inhibitory Concentration of *Excoecaria agallocha* leaves extract

Methanol Extract (μg/ml)	Bacterial Growth
31.25	++++
39	++++
46.90	+++
62.50	+++
78.13	+++
93.75	++
125	++
156.25	++
187.50	++
250	++
312.50	+
375	+
500	-
625	-
750	-
1000	-
1250	-
1500	-
2000	-
2500	-
3000	-

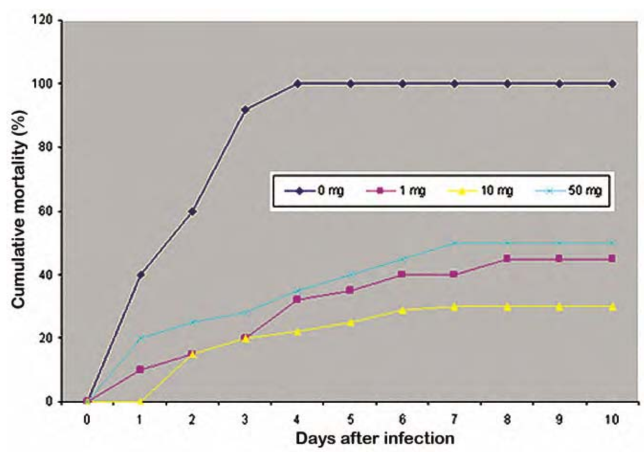

Fig. 3—Effect of different dosage of *Excoecaria agallocha* leaves extract on the cumulative percent mortality (%)

compounds, proteins, free amino acids, gums, mucilage, flavonoids, lignin and absence of carbohydrates, glycosides, saponins and volatile (Table 3).

Discussion

Marine aquarium keeping is an emerging field and ornamental fishes have very good marketable demand. Bacterial diseases are the most common infectious problem during culture time. Treatment of marine ornamental fish disease has not yet been studied well. In the present study, a trail has made to rear the disease free fishes with the sustainable components. In continuation, the bacteria have isolated from infected common clownfish and identified as *Aeromonas hydrophila*. The result is in lined with the reports which revealed that the Gram-negative organisms cause the majority of bacterial infections including the following pathogenic genera: *Aeromonas, Citrobacter, Edwardsiella, Flavobacterium (flexibacter), Pseudomonas,* and *Vibrio*. Some literature reported that *Vibrio alginoluticus*, *Pseudomonas* sp., *Aeromonas hydrophila* are the predominant bacterial pathogens causing diseases in ornamental fishes[21].

Since, antibiotic resistance can cause significant danger and create serve problem in treatment process, microorganisms can develop resistance to specific antibiotics. *A. hydrophila* also found to be resistance against most of the antibiotics used in this study. It indicated that the bacterial pathogens are creating a resistance may due to transposable elements, conjugation between organisms, transformation and mutation[22].

The methanol and acetone extract of *E. agallocha* leaves were studied against bacterial isolates. The obtained results are in lined with various scientific reports[23,24]. Earlier studies expressed that the extract of *E. agallocha* exhibited significant *in vitro* antibacterial activity against *Staphylococcus aureus*, *Shigella dysenteriae, Shigella sonnei, Aeromonas hydrophila, Pseudomonas fluorescens* and *Enterococci* sp[10-12]. In the present study, minimal inhibitory concentration of *E. agallocha* was found between 500 µg/ml. Previous reports indicated that the *E. agallocha* contains salts, organic acids, carbohydrates, hydrocarbons, benzoquinone, naphthofurans, sesquiterpenes, triterpenes, alkaloids, flavonoids, polymers, sulfur derivatives and tannins[25,26]. In the present findings also recorded the presence of alkaloids, phytosterols, fixed oils, fats, tannins, phenolic compounds, proteins, free amino acids, gums, mucilage, flavonoids, lignin and absence of carbohydrates, glycosides, saponins, volatile. After the challenge experiment with *A. hydrophila*, all the treated groups have reduced mortality compared to the control group. The best survival rate was observed in the group treated with methanol extract. The mortality observed following challenge with *A. hydrophila* was decreased in the group of fish treated with mangrove extract. Compared with untreated controls, methanol extracts treatment increased the survival rate of the fish infected with *A. hydrophila*, with a minimum of 30% in the 10 mg kg $^{-}$1 group and the maximum of 45% in the 1 mg kg $^{-}$1 group. Earlier studies in this line also revealed that dietary supplementation of *O. sanctum*[27] intraperitoneal injection of water soluble and hexane soluble fraction of *Eclipta alba* have enhanced the disease resistance against *A. hydrophila* in *Oreochromis mossambicus*. The present finding is in agreement with previous studies in the Tilapia fish fed[28] with a diet containing ethyl acetate extract of *Rosmarinus officinalis* leaf powder conducted a study[29] in carp administered with the fraction II of *Undaria pinnatifida*. They also found that the administration of this fraction 6 and 3 days prior to intraperitoneal challenge with *Eclipta tarda* significantly increased the survival rate. The disease resistance against *A. hydrophila* was enhanced in *L. rohita* fed with 0.5% of Achyranthes[30]. It is important to estimate the increased protection in the methanol extract treated fish. In this study, after challenge with *A. hydrophila*, all dosage groups showed a reduced mortality compared to the control group. These results indicated that methanol extracts

Table 3 - Phytochemical screening of of *Excoecaria agallocha* leaves extract

S.No	Phytoconstituents	Methanol
1	Alkaloids	+
2	Carbohydrates	-
3	Glycosides	-
4	Phytosterols	+
5	Saponins	-
6	Fixed oils & Fats	+
7	Tanins & Phenolic compounds	+
8	Proteins & free amino acids	+
9	Gums & Mucilage	+
10	Flavonoids	+
11	Lignin	+
12	Volatile	-

have some ability to activate the immune system of common clownfish, *A. sebae*.

To summarize, the results of our study showed that *E. agallocha* leaves could significantly enhance the survival of common clownfish. The lowest mortality was observed in the group treated with 10 mg kg^{-1} doses of methanol extracts in the disease resistance against *A. hydrophila*. In conclusion, the administration of methanol solvent extract enhanced disease resistance against *A. hydrophila* in Common clownfish. Also further investigation on the immunostimulatory effect of mangrove extract for disease prevention in ornamental aquaculture is warranted.

Acknowledgments

Authors are thankful to the authorities of the Annamalai University for providing facilities and the University Grants Commission (UGC), New Delhi for financial support.

References

1 Ajith Kumar T T, Nithya Jeniffer P, Murugesan P & Balasubramanian T, Marine Ornamentals in India: Challenges and Opportunities for Sustainability. *Fishing chim.*, 27 (2007) 44-51.

2 Karunasagar I, Rosalind G & Karunasagar J, Immunological response of the Indian major carps to *Aeromonas hydrophila* vaccine. *J. Fish Dis.,* 14 (1991) 413-417.

3 Hazen T C, Fliermans C B, Hirsch P & Esch G W, Prevalence and distribution of *Aeromonas hydrophila* in the USA, *J. Appl. Environ. Microbiol*., 36 (1978) 731-738.

4 Amin N E, Abdallah I S, Elallawy T & Ahmed S M, Motile *Aeromonas septicaemia* among *Tilapia nilotica* (*Sarotherodon niloticus*) in upper Egypt, *Fish Pathol.,* 20 (1985) 93-97.

5 Miyazaki M & Jo S, A histopathological study of motile aeromonad disease in Ayu *Plecoglossus altivelis*. *Fish Pathol*., 20 (1985) 55-60.

6 Harikrishnan R, Nisha Rani M & Balasundaram C, Hematological and biochemical parameters in common carp, *Cyprinus carpio*, following herbal treatment for *Aeromonas hydrophila* infection. *Aquacul.,* 221(2003) 41-50.

7 Rijkers G T, Teunissen A G, Van Oosteron R & Van Muiswinkel WB, The immune system of cyprinid fish. The immuno-suppressive effects of the antibiotic oxytetracycline in carp (*Cyprinus carpio* L.). *Aquacul.,* 19 (1980) 177-189.

8 Robertsen B, Modulation of the non-specific defence of fish by structurally conserved microbial polymers, *Fish Shellfish Immunol*., 9 (1999) 269-290.

9 Afidah A, Rahim, Emmanuel Rocca, Jean Steinmetz, Jain Kassim M, Sani Ibrahim M & Hasnah Osman, Antioxidant activities of mangrove *Rhizophora apiculata* bark extracts, *Food Chemistry* 107 (2008) 200-207

10 Kathiresan K, A review of studies on Pichavaram mangroves, Southeast India, *Hydrobiol.,* 430 (2000) 185-205.

11 Premanathan M, Arakaki R, Izumi H, Kathiresan K, Nakano M & Yamamoto N, Antiviral properties of a mangrove plant, *Rhizophora apiculata* Blume, against human immunodeficiency virus. *Antiviral Res.*, 44 (1999) 113-122.

12 Kathiresan K, Sithranga Boopathy N & Kavitha S, Coastal vegetation: An underexplored source of anticancer drugs, *Nat. prod. rad.,* 5 (2000) 115-119.

13 Kumaran S, Deivasigamani B, Alagappan K M, Sakthivel M, Guru Prasad S, Isolation and characterization of *Pseudomonas* sp. KUMS3 from Asian sea bass (*Lates calcarifer*) with fin rot, *World J Microbiol Biotechnol*., 26 (2010) 359-363

14 Cappuccino G & Sherman N, Microbiology: a laboratory manual. *Benjamin/Cumming Science Publishing*, California, (1998) 254

15 Premanathan M, Rajendran S, Ramanathan T, Kathiresan K, Nakashima H & Yamamoto N, A survey of some Indian medicinal plants for anti-human immunodeficiency virus (HIV) activity, *Indian J. Med. Res*., 112 (2000) 73-77.

16 Bauer A W, Kirby W M M, Sherris J C & Truck M, Antibiotic susceptibility testing by a standardised single disk method. *American J. Clinical* Pathol., 45 (1966) 493-496.

17 NCCLS. Performance standards for antimicrobial disk susceptibility test - 3rd Eds., Approved standards, National Committee for Clinical Laboratory Standards: M7-A3, Villa Nova, P.A. (1993).

18 Evans W C, An index of medicinal plants. *A Text book of Pharmacognosy,* 14 ed. 7(1997) 12-14.

19 Harikrishnan R, Balasundaram C, Man-Chul K, Ju-Sang K, Yong-Jae H & Moon-Soo H, Innate immune response and disease resistance in *Carassius auratus* by triherbal solvent extracts, *Fish & Shellfish Immunol.,* 27 (2009) 508-515.

20 Dhayanithi N B, Ajith Kumar T T & Kathiresan K, Effect of neem extract against the bacteria isolated from marine fish, *J. Environ. Biol., 31* (2010) 409-412.

21 Choudhury S, Sree1 A, Mukherjee S C, Pattnaik P & Bapuji M, *In vitro* Antibacterial Activity of extracts of selected marine algae and mangroves against fish pathogens. *Asian Fish. Sci.*, 18 (2005) 285-294.

22 Rani P & Khullar N, Antimicrobial evaluation of some medicinal plants for their anti-enteric potential against multi-drug resistant *Salmonella typhi*, *Phytother. Res*., 18 (2004) 670-673.

23 Peter K L N & Sivasothi N A, Guide to the Mangroves of Singapore I: The Ecosystem and Plant Diversity, Singapore Science Centre, (1999)111-112.

24 Ravikumar S, Gnanadesigan M, Suganthi P & Ramalakshmi A, Antibacterial potential of chosen mangrove plants against isolated urinary tract infectious bacterial pathogens, Int. J.Medicine & Med.Sci., 2 (2010) 94-99.

25 Konishi T, Yamazoe K, Kanzato M, Konoshima T & Fujiwara Y, Three diterpenoids (Excoecarins V1-V3) and a flavanone glycoside from the fresh stem of *Excoecaria agallocha*, *Chem. & Pharm. Bull.*, 51 (2003) 1142-1146.

26 Ericson K L, Beutler J A, Cardellina J H, McMahon J B, Newman J D & Boyd M R A, Novel phorbol ester from *Excoecaria agallocha*, J. Nat. Prod,. 58 (1995) 769-72.

27 Logambal S M & Michael R D, Azadirachtin - an immunostimulant for *Oreochromis mossambicus* (Peters). *J. Aqua. Trop.,* 16 (2001) 339-347.

28 Abutbul S, Golan-Goldhirsh A, Barazani O & Zilberg D, Use of *Rosmarinus officinalis* as a treatment against *Streptococcus iniae* in tilapia (*Oreochromis* sp.), *Aquacul.,* 238 (2004) 97-105.

29 Fujiki K, Matsuyama H & Yano T, Protective effect of sodium alginates against bacterial infection in common carp *Cyprinus carpio* L, *J. Fish Dis.,* 17 (1994) 349-55.

30 Joseph S W & Carnahan A, The isolation, identification, and systematics of the motile *Aeromonas* species, *Annu. Rev. Fish Dis.,* 4 (1994) 315-43.